# Investigating the Interactions Between Protocadherin-19 and Ryk and its Effect on Neurogenesis

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**ABSTRACT**

Protocadherins are a large, diverse family of neural cell adhesion proteins, but their functions are not understood. Mutations or in several protocadherins have been associated with neurological disorders. For example, Protocadherin19 (*PCDH19*) causes epilepsy in females with mental retardation (EFMR). In this X chromosome-linked disease, girls randomly express one good allele or one mutant allele per cell due to a phenomenon known as X-linked inactivation. This mosaic expression leads to the symptoms classified as EFMR, but the effects on cellular pathways for the disease are not known. In zebrafish, the loss of Pcdh19 leads to the loss of columnar organization in the developing optic tectum through the loss of adhesion and an increase in neuronal proliferation and differentiation. The literature shows that Pcdh19 interacts with Ryk, a noncanonical Wnt receptor involved in neurogenesis. Based on previous data and the literature, we hypothesize that Pcdh19 binds to the extracellular domain of Ryk to inhibit binding of the Wnt3 ligand, preventing the intracellular domain of Ryk from being cleaved and translocated to the nucleus where it would initiate pathways for neuronal proliferation. Immunohistochemistry and co-immunoprecipitation were attempted to show if Pcdh19 affects cleavage of the Ryk intracellular domain, its nuclear translocation, and subsequent Wnt signaling. Transgenic and Ryk-knockout fish are being developed to perform experiments *in vivo*.

## PROBLEM STATEMENT

The developing embryo requires many processes to build a mature brain such as neurogenesis, neurulation, patterning of brain regions, neuronal or glial differentiation, neuronal migration, axon guidance, and synaptogenesis (Weiner and Jontes, 2013). All of these processes are controlled by a myriad of mechanisms, of which many work synchronously to accomplish a particular task. These neurodevelopmental events accumulate to produce functional neural circuits in the central and peripheral nervous systems that control cognition, emotion, behavior, and homeostasis. These processes must be tightly controlled to ensure that the neurons synapse on the correct partners. If any of these mechanisms are disrupted even slightly, the end result can be minor defects that may manifest themselves as small behavioral quirks, or they can be catastrophic morphologies that lead to miscarriages. However, not all disastrous breakdowns in neurodevelopment lead to a miscarriage. Other developmental malfunctions can cause moderate to severe cognitive and behavioral disabilities, but the child grows to adulthood due to the low lethality of the disorder. In fact, many neurological disorders can be categorized as neurodevelopmental issues, even if the disease doesn't occur until later in life.

One neurological disorder that has become of interest in recent years is epilepsy in females with mental retardation (EFMR). EFMR is a neurodevelopmental disorder characterized by seizures and impaired intellectual functioning in females and starts presenting symptoms at a young age (Stevenson, 2012). Other psychiatric disorders such as obsessive and autistic behaviors have been linked to EFMR as well (Scheffer, et al., 2008). The symptoms of EFMR resemble Dravet Syndrome, a severe type of genetic epilepsy, and ranks second in prevalence of genetic epileptic disorders (Kwong, et al., 2007; Scheffer, 2012). Previous research has shown that heterozygous inheritance of a mutation in the Protocadherin-19 (*PCDH19*) allele leads to the

symptoms demonstrated in EFMR (Depienne et al., 2011). Strangely, homozygous inheritance of the correct or mutant allele does not lead to EFMR; only heterozygous inheritance of a mutation in *PCDH19* leads to symptoms. Since *PCDH19* resides on the female sex chromosome, the suspected cause for this unusual phenotype is X-chromosome inactivation (Duszyc, 2015). The stochastic expression of the normal allele in some cells and the mutant allele in others could disrupt the neural circuitry and create a form of miscommunication in the brain (Wu, et al., 2014). However, the molecular mechanisms that would be affected as a result of X-chromosome inactivation are unknown. Defining the mechanism of EFMR would help doctors and researchers treat patients more effectively, could be extrapolated to understand other forms of epilepsy, and could be used to understand other neurological diseases as a result of X-chromosome inactivation.

Pcdh19 is a member of the protocadherin subfamily which is a part of the cadherin superfamily of adhesion molecules. Cadherins comprises approximately 100 members, with protocadherins consisting of at least 60 (Kim, et al., 2011; Redies, et al., 2012; Weiner and Jontes, 2013). Many of these proteins are expressed in the nervous system. Due to their diversity, similarity to classical cadherins, and different spatiotemporal expression, some investigators predict that protocadherins encode for particular circuits with stereotyped functions (Hertel, et al., 2008; Krishna, et al., 2011; Redies, et al., 2011; Vanhalst, et al., 2005). If this hypothesis is true, it could revolutionize our understanding and treatment of neurodevelopmental disorders. Therefore, study of protocadherins, specifically Pcdh19, outside of the context of disease is also critical to comprehending the development of the brain and how it forms neural circuits.

## BACKGROUND

As mentioned earlier, protocadherins are a subfamily of the cadherin family of cell-adhesion molecules. These proteins have been organized into one category based on the presence of repeated cadherin domains on the extracellular surface (Weiner and Jontes, 2013). However, their differing properties and sequence conservation has led to classifying cadherins in several subfamilies (Redies, et al., 2005). Protocadherins contain 6-7 cadherin repeats, expressed principally in the nervous system, and are organized into clustered and non-clustered protocadherins along with a couple of smaller groups (Kim, et al., 2011; Vanhalst, et al., 2005; Weiner and Jontes, 2013). Clustered protocadherins all appear near each other on the genome, whereas the non-clustered protocadherin genes are scattered (Kim, et al., 2011).

Within the group of non-clustered protocadherins, there are delta protocadherins. They also have highly conserved motifs in the cytoplasmic domain (Kim, et al., 2011). Pcdh19 falls under the domain of delta protocadherins. Generally, protocadherins exhibit weak, homophilic adhesion, unlike classical cadherins (Emond, et al., 2011; Kahr, et al., 2013). However, cis-complexes between protocadherins and other molecules help to enhance protocadherin-mediated adhesion (Emond, et al., 2011; Weiner and Jontes, 2013). As described earlier, protocadherins are theorized to be involved in neural circuit formation. In fact, clustered protocadherins have been shown to be involved in dendrite arborization, axon guidance, synaptogenesis, and apoptosis (Weiner, 2013; Redies, 2012). Similarly, non-clustered protocadherins have been implicated in cell fate, axon guidance, cellular motility, synapse formation and stability (Kahr, 2013; Redies, 2012). Additionally, classical cadherins can modulate the adhesive capability of delta protocadherins, further suggesting that non-clustered protocadherins are involved in circuit formation in the nervous system (Kim, 2011).

Unlike some delta protocadherins, Pcdh19 has been directly connected to a neurological disease, which has been labeled EFMR (Camacho, et al., 2012). As mentioned earlier, this disease has puzzled researchers. Most X-linked mutations lead to symptoms in males because they only have one copy of genes which make them susceptible to both dominantly and recessively inherited diseases (Dibbens, et al., 2008). With only one X-chromosome, there isn't the possibility for another healthy copy of the gene to compensate. Strangely, males that have or inherit a mutated and previously pathogenic form of the gene do not demonstrate symptoms (Depienne and LeGuern, 2012). Females are subject to X-linked dominant and recessive disorders. However, females that have mutations in both alleles of Pcdh19 do not show symptoms (Redies, et al., 2012; Harssel, et al., 2013). This genetic scenario would lead to the expression of dominant and recessive X-linked diseases, but this doesn't actually occur. EFMR only occurs when a female has one correct copy and one mutated copy. The resulting hypothesis is that X-linked activation within among the neurons leads to scrambled communication, also known as cellular interference (Redies, et al. 2012; Harssel, et al., 2013; Dibbens, et al. 2008; Leonardi, et al., 2014). A couple of studies reported a few male patients with EFMR symptoms and mosaicism of de novo mutations in *PCDH19* (Depienne, et al., 2009; Terracciano, et al., 2016). These findings further support the hypothesis of X-linked inactivation leading to interference amongst neurons. However, the pathogenic mechanism resulting from X-linked inactivation of a heterozygous female is unknown. Research suggests an inflammatory component due to the effectiveness of corticosteroids, but this has yet to be corroborated (Higurashi, et al., 2015).

As the name suggests, EFMR is primarily characterized by epilepsy and cognitive defects in females. Onset of seizures usually happens somewhere between 9 and 36 months with an

average onset at one year of age (Leonardi, et al., 2014; Higurashi, et al., 2013; Depienne and LeGuern, 2012). Seizures are often elicited by fevers (Specchio, et al., 2011). EEGs of EFMR patients are very similar to EEGs of patients diagnosed with Dravet syndrome. As a result, researchers often find EFMR patients among populations with Dravet-like symptoms but no mutations in the SCN1A gene (Kwong, et al., 2012; Scheffer, 2012). The characteristics of the seizures vary, but they are often described as clustered, focal seizures that can later develop into generalized seizures. Often accompanied by fearful screaming, EFMR patients demonstrate many types of seizures, such as tonic, clonic, tonic-clonic, myoclonic, absent, and atonic, which indicates that genetic screening is necessary for a definitive diagnosis (Higurashi, et al., 2013; Marini, et al., Marini, et al. 2010; 2011; Scheffer, et al., 2008; Specchio, et al. 2011). Early in life, infants and toddler experience 1-5 minute seizure up to 10 times a day. As they age, the severity and frequency of seizures decreases. Seizures disappear for some patients during adolescence, but they can persist throughout life for others (Higurashi, et al., 2013; Marini, et al., 2010; Specchio, et al., 2011; Scheffer, et al., 2008).

Once the seizures begin, many patients begin to develop cognitive and behavioral defects. About 30% of patients retain normal intelligence, but the remaining 70% demonstrate mild to severe intellectual impairment (Dibbens, et al., 2008; Higurashi, et al., 2013; Marini, et al., 2012; Scheffer, et al., 2008). Several studies found patients with autistic, aggressive, and obsessive behaviors (Camacho, et al., 2012; Depienne and LeGuern, 2012; Scheffer, et al., 2008). Unlike the epileptic episodes, cognitive and psychiatric disabilities do not disappear with age. Females diagnosed with EFMR are linked by a mutation in the *PCDH19* gene and onset of epilepsy as an infant. However, the variability in the type and severity of the seizures, intellectual impairment, and psychiatric disorders suggests two possibilities, of which one or both may be true. The first

scenario is that *PCDH19* expression may be significant enough where disruptions lead to a maladaptive phenotype, but its expression may not be so ubiquitous that a normal phenotype cannot be recovered. Differential expression in distinct brain regions may prevent the onset of catastrophic symptoms such as those in other genetic disorders. The other scenario is that *PCDH19* is part of a highly intricate system with many compensatory mechanisms that help mitigate symptoms and may even eliminate epileptic episodes.

However, most research into EFMR simply describes novel mutations in *PCDH19*, develops pedigrees, and characterizes the symptoms of disease (Camacho, et al., 2012; Leonardi, et al., 2014; Redies, et al., 2012; Harssel, et al., 2013; Dibbens, et al., 2008; Marini, et al., 2010; Higurashi, et al., 2013). The only results that have been any indicator of a molecular mechanism are that most pathogenic mutations occur in the extracellular domain and that corticosteroids alleviate epileptic seizures in EFMR patients (Harssel, et al., 2013; Higurashi, et al.; 2015; Depienne and LeGuern, 2012). Otherwise, no literature was found that describes a molecular mechanism for the disease. Our lab has turned its focus toward Pcdh19 to understand the roles of protocadherins in the nervous system.

Due to characteristics of embryonic and larval development, *Danio rerio* has become a well-established model for neural development (Rinkwitz, et al., 2011). Although zebrafish does not develop a neocortex analogous to mammals, many genes, proteins, and developmental pathways are conserved or very similar to those in mammalian pathways (Panula, et al., 2010). For example, the optic tectum, the primary visual processing center for zebrafish, develops in a fashion similar to the cortex, although it does not form the same cortical structure (Sumbre and de Polavieja, 2014). Progenitors in the neural epithelium along the ventricular zone divide into cells that will differentiate into neurons and glia. Radial glia extend processes to the pial surface

and freshly-formed neurons climb up the processes into their mature positions where they form layers in the optic tectum (Nadarajah, et al., 2001). This process also occurs in mammals. We wanted to study how Pcdh19 would affect mechanisms related to the formation of cortex in mammals. We hypothesized that Pcdh19 would be important for adhering developing neurons to radial glia so they can reach the correct position.

Using a 5xUAS-GAL4 system to express LifeAct in Pcdh19-expressing neurons in wild type zebrafish shows that Pcdh19-expressing neurons associate in distinct radial columns during neurodevelopment (Fig 1A, D). CRISPR RNAs were injected in zebrafish that express the LifeAct reporter in Pcdh19-expressing cells at one cell stage to make Pcdh19 knockouts and later stages to make Pcdh19 knockdowns simulating the mosaic expression that would result from X-

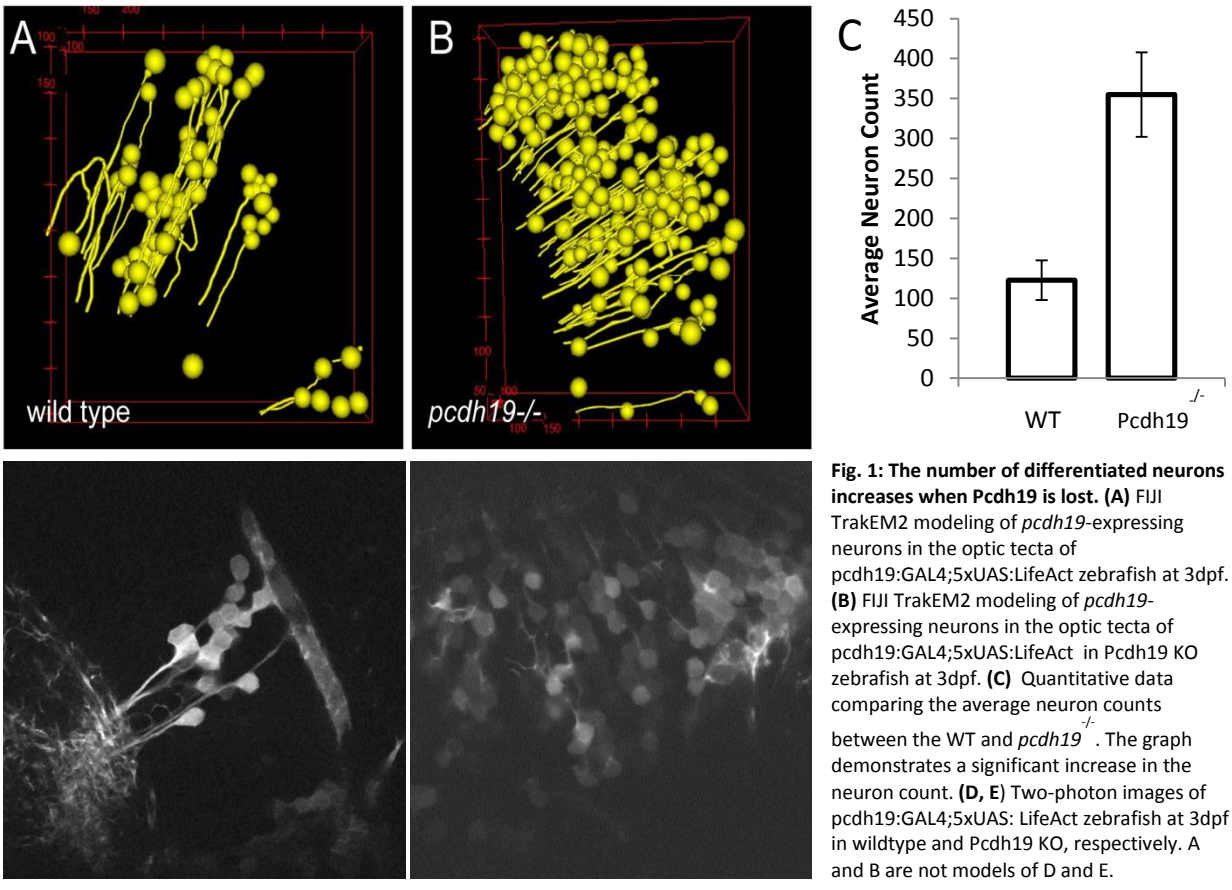


**Fig. 1: The number of differentiated neurons increases when Pcdh19 is lost. (A)** FIJI TrakEM2 modeling of *pcdh19*-expressing neurons in the optic tecta of pcdh19:GAL4;5xUAS:LifeAct zebrafish at 3dpf. **(B)** FIJI TrakEM2 modeling of *pcdh19*-expressing neurons in the optic tecta of pcdh19:GAL4;5xUAS:LifeAct in Pcdh19 KO zebrafish at 3dpf. **(C)** Quantitative data comparing the average neuron counts between the WT and *pcdh19*$^{-/-}$. The graph demonstrates a significant increase in the neuron count. **(D, E**) Two-photon images of pcdh19:GAL4;5xUAS: LifeAct zebrafish at 3dpf in wildtype and Pcdh19 KO, respectively. A and B are not models of D and E.

inactivation of Pcdh19. Behavioral data of mosaics showed that zebrafish demonstrated significant impairment of visual recognition and reaction while wild type and complete Pcdh19 knockouts did not. Two-photon imaging of LifeAct, which targets GRP to F-actin, in mutants lacking Pcdh19 showed loss of columnar organization. There was also increased neurogenesis and an increase in the number of neurons expressing Pcdh19 (Fig 1B, E). The mechanism for these observations is not known.

Numerous signaling pathways through diffusible morphogens or cell-to-cell contacts are involved in neuronal differentiation or neurogenesis, such as hedgehog and Notch/Delta signaling pathways (Hatakeyama, et al., 2014; Bambakidis, et al., 2012). The Wnt signaling pathway is also very important for normal development of nervous tissue. In humans, there are nineteen genes that code for Wnt glycoproteins and the ten receptors in the Frizzled family of G-protein coupled receptors that bind to those ligands (Angers and Moon, 2009). This allows the Wnt signaling pathway to be involved in numerous developmental mechanisms including neurogenesis, axial patterning, neuronal migration, and cellular differentiation (Zhong, 2008).

Wnt signaling is also categorized into beta-catenin dependent and beta-catenin independent. Wnt/beta-catenin signaling is the best known pathway and is classified as the canonical Wnt pathway, although there are several noncanonical transduction mechanisms (MacDonald, et al., 2009). Without Wnt to bind to in the extracellular fluid, beta-catenin is phosphorylated by a complex involving Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3),and casein kinase 1α (CK1α) (Kikuchi, 1999). Phosphorylated beta-catenin is then ubiquitinated by SKP1–cullin 1–F-box E3 ligase for degradation in the proteasome (Angers and Moon, 2009). In other words, lack of Wnt signaling leads to degradation of beta-catenin in the cytoplasm. When a Wnt glycoprotein binds to a Frizzled

receptor, it recruits low density lipoprotein receptor-related protein 5 or 6 (LRP5 or LRP6) into a complex on the membrane (Kikuchi, 1999). Recruitment of Dishevelled protein to the Frizzled receptor, and of GSK3-beta to the LRPs leads to phosphorylation of the LRP and binding of Axin (Gao and Chen). With Axin in a complex with LRP5 or LRP6, the degradation complex is inactivated, and beta-catenin begins accumulating in the cytoplasm and eventually translocates to the nucleus where it binds to TCF/LEF family of transcription factors to stimulate transcription of genes involved in several processes (MacDonald, et al., 2009; Angers and Moon, 2009).

Another important Wnt receptor is Ryk. Ryk is consists of an extracellular domain (ECD), transmembrane domain, and intracellular domain (ICD). The extracellular domain uses a WIF-type Wnt binding domain. The intracellular domain is a tyrosine kinase that lost its intrinsic kinase capabilities (Lu, et al., 2004). Instead, the intracellular domain is cleaved by gamma-secretase in neural progenitors, and translocation of the ICD to the nucleus leads to activation of Wnt target genes (Lyu, et al., 2008). Ryk is involved in activating beta-catenin independent pathways when interacting with the Wnt5 and Wnt11 ligands, which regulates axon guidance and cell migration (Berndt, et al., 2011). Ryk also binds Wnt3a to activate Wnt/beta-catenin leading to neuronal proliferation and differentiation (Lyu, et al., 2008). Ryk is regulated by the activity of MIB1, an E3 ubiquitin ligase. MIB1 ubiquitinates Ryk to promote degradation, but it can also form a complex with Ryk to activate Wnt/beta-catenin signaling (Berndt, et al, 2011).

Ryk's involvement in Wnt signaling is intriguing because Berndt, et al. (2011) conducted a tandem affinity purification of Ryk along with mass spectrometry. They identified numerous cellular adhesion proteins in the cadherin superfamily, including several members of protocadherins. Protocadherin-19 was one of the binding partners of Ryk, including other protocadherins (Berndt, et al., 2011; Kahr, et al., 2013). Additionally, other members of the

cadherin superfamily, including protocadherins, have been shown to interact with proteins known to be parts of the Wnt signaling pathways (Kahr, et al., 2013; Kim, et al., 2011; Redies, et al., 2005; Weiner and Jontes, 2013). This suggests that protocadherins have a legitimate role in Wnt signaling. We performed a tandem affinity purification of Pcdh19-TAP and Ryk-GFP. We confirmed that Ryk and Pcdh19 formed a complex. Given our data showing zebrafish with Pcdh19 knocked down have increased numbers of neurons and increased numbers of Pcdh19-expressing neurons, it is possible that Wnt3a binding to Ryk regulates proliferation and differentiation of Pcdh19-expressing neurons. Since the presence of Pcdh19 in wild type fish demonstrate a lower cell count and less Pcdh19 differentiation, Pcdh19 may interact with Ryk to inhibit Wnt signaling.

## METHODS

### *Genetic Recombineering*

Ryk cDNA for Danio rerio was obtained by RT-PCR from 48 hpf embryos. Restriction enzymes were used to insert the cDNA into pEGFP-N1, which is driven by the CMV promoter. Plasmids were transformed into TOP10 cells and later concentrated to be used in in vitro experiments and embryonic injections. BAC clone CH211-66i8 was obtained from BACPAC resources. BAC clone CH211-66i8 contains the full Ryk genomic locus, and itol2 and Gal4 were subsequently added.

### *PCR Site Mutagenesis*

Primers were designed to create PCR products that split the transmembrane and extracellular domain from the intracellular domain. The design for the primers are listed as follows: RykDN-BamHI-R: 5’-CGCGGATCCCCAC…ATGCTGTGGAGT-3’, which inserted a

BamHI site at the end of the extracellular domain of Ryk; RykICD-XhoI-F: 5'-CGCCTCGAGGCCA…AATGGATGACAGC-3' which inserted a XhoI site in front of the intracellular domain of Ryk; RykFix-F: 5'-ATTCACAAAGACTTGGCTGCC-3'; RykFix-R: 5'-CAAGTCTTTGTGA…TGACCTCTCTCCG-3' to fix the mutation in the gene.

*CRISPR*

Clustered regularly-interspaced short palindromic repeats (CRISPR) were designed against several segments in exon 1 of Ryk to create a transgenic Ryk knockout. Sites were selected directly upstream of a NGG PAM site. Single stranded antisense oligos were ordered from, annealed using PCR, and then TOPO cloned into a pDR274 plasmid vector. The CRISPR was designed against the sequence, 5'-GCGGCGTCGGCGGTGTCCCGGGG-3'. The two antisense oligos were 5'-TAGCGGCGTCGGCGGTGTCCCGGGG-3' and 5'-AAACCCCCGGGACAC CGCCGACGCC-3'. CRISPR guide RNAs were transcribed using the MAXIscript ® T7 kit by Ambion and Cas9 RNA was transcribed using the mMESSAGE mMACHINE kit by Ambion. Guide RNAs and Cas9 RNAs were injected in 1-cell and 2-cell stage embryos.

*Genotyping*

CRISPR sites were strategically chosen because of the existence of a SmaI restriction site at the first cut sequence. CRISPR-injected zebrafish were screened by digesting embryos and tailfins in 100 uL 50mM NaOH for 10 minutes at 95°C. Then, 10 uL of 1M Tris pH 8 was added to neutralize the digestion. PCR of the digested DNA was performed, followed by the appropriate restriction digest, and then analyzed on an agarose gel. Two cut bands indicated that the CRISPR didn't work, but a protected band indicated that it was a success. Primers for PCR of the NaOH-digested DNA were as follows: Ryk-test-F 5'-TTTTGGATGAGGTTCCGTGG-3'

and Ryk-test-R 5'-GTTTCTTGACCTCTTCCTCG-3'. High resolution melt analysis was also performed to corroborate restriction digest results. Additional primers were designed for a smaller amplicon. Primers designed around the CRISPR site for HRMA were 5'-TCCACAGGCCATCTCTCAGC-3' and 5'-GTCTGTGATCTTGACTTGCATGC-3'.

*Zebrafish Maintenance*

Zebrafish were maintained at 28°C while being fed twice daily with fish food and brine shrimp. Wild type and transgenic lines were crossed according to the method detailed by Westerfield (1995). Embryos were collected and grown in E3 embryo media in a 28°C incubator for one week, then transferred to fish tanks.

*Cell Culture*

HEK293 cells were obtained from ATCC. Cells were cultured in Dubecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS). At 80% confluence, cells were transfected using a calcium-phosphate transfection method. 2xHBS was added to an equal volume of water, DNA (4% by mass), and 2M $CaCl_2$ (12.2% by volume). The following day cells were used.

*Co-immunoprecipitation*

Transfected HEK cells were lysed in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, and protease inhibitor from Roche). Lysates were incubated with the appropriate GFP, HA, or IgG beads overnight at 4°C. Lysates were then centrifuged at 4°C for 10 min, and identical volumes were loaded into two wells per condition onto NuPAGE 10% Bis-Tris SDS-PAGE gels purchased from Life Technologies. After electrophoresis, proteins were transferred to PVDF membrane from GE Healthcare in transfer buffer from Bio-Rad laboratories. Blots were blocked with 5% nonfat milk in TBST (10 mM Tris-HCL, pH 7.6, 100

mM NaCl, and 0.1% Tween-20) and incubated overnight at 4°C with primary antibody (rabbit anti-GFP from Life Technologies at 1:1250; mouse anti-HA at 1:5000; mouse anti-strep tag from Millipore at 1:2500; mouse anti-myc from Millipore at 1:2500; mouse anti-V5 from Genscript at 1:2500). Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories were added at a concentration of 1:5,000. PerkinElmer's Western Lightning Ultra kit was used to develop a chemiluminescent signal on the blots, which were imaged using the UltraLum imaging system.

*Immunocytochemistry*

Transfected HEK293 cells were fixed for 10 mins in 4% paraformaldehyde in PBS. Cells were washed in PBS then permeabilized with 0.025% Triton X-100 in PBS. Cells were then blocked overnight in PBS, 0.5% Triton X-100, 1% DMSO, 10% normal goat serum and primary antibody at a dilution of 1:50 antibody (rabbit anti-GFP from Life Technologies; mouse anti-HA; mouse anti-strep tag from Millipore; mouse anti-myc from Millipore). Cells were then blocked in PBS, 0.5% Triton X-100, 1% DMSO, 10% normal goat serum and anti-mouse and anti-rabbit secondary antibody Dylight 488 and 549 at a dilution of 1:50 antibody. Fluoromount G was placed on microscope slides, and the coverslips were placed on the Fluoromount G to preserve cells for future imaging. Phosphone-histone3 staining was conducted to measure proliferation. Wild type and mutant larva at various time points were fixed in 4% paraformaldehyde in PBS. They were permeabilized in PBS + 0.25% Triton X-100 and later incubated in block (PBS, 1.5% BSA, 2% NGS, PBS) with α-PH3 antibody from Cell Signaling. The samples were later blocked with secondary α-rabbit Dylight 488 antibody for visualization.

*Two-photon microscopy and image analysis.*

Two-photon imaging fixed zebrafish at 24 hpf was performed at room temperature on a custom-built microscope. Excitation was provided by a Chameleon-XR Ti :Sapphire laser (Coherent). Fluorescence was detected using a Multiphoton Detection Unit mounted on a SliceScope (Scientifica). Water immersion objectives from Olympus were used, either 60×/NA1.0 (LUM PLFLN60X/W) or 20×/NA1.0 (XLU MPLFLN20XW). Image analysis was performed with Fiji. Stack of approximately 40 optical sections were imaged from WT or Pcdh18b mutants. Stacks were assembled into 3D images so that positive cells could be counted.

**RESULTS**

Before experiments could be conducted, the Ryk gene for *Danio rerio* had a point mutation that resulted in a lysine to glutamate transition at the 460th amino acid. Primers were purchased to perform PCR site mutagenesis to un-mutagenize the gene, which was completed after several attempts. Lyu et al. (2008) showed that Ryk transduced its signal through the cleavage of the intracellular domain and its translocation to the nucleus to initiate genes involved in neuronal differentiation. To further understand the mechanism and Pcdh19's effect on Ryk-mediated Wnt signaling, PCR site mutagenesis was again performed to create a dominant-negative Ryk (RykDN) and an intracellular domain of Ryk (RykICD). RykDN consisted of the extracellular WIF binding domain and transmembrane domain so that it remained on the plasma membrane. RykDN could bind any potential ligand or co-receptor, but does not transmit a signal. RykICD contains the nonfunctional tyrosine kinase domain, undergoes nuclear translocation, and initiates transcription.

Berndt, et al (2011) demonstrated that Ryk formed a complex with many proteins, including Pcdh19 and other protocadherins. We wanted to confirm that Ryk actually interacted

with Pcdh19 through coimmunoprecipitation. HEK293 cells were cultured and transfected with plasmids containing Pcdh19-TAP and GFP-conjugated Ryk constructs. Staining on blots showed

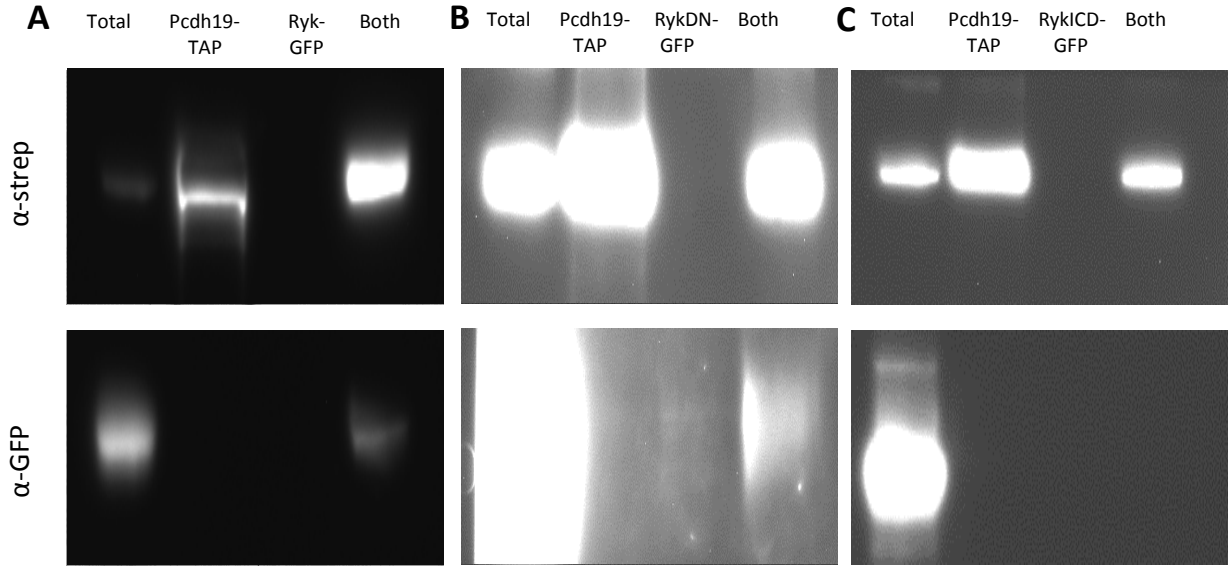


**Fig. 2** Coimmunoprecipitations of Pcdh19-TAP and Ryk-GFP (A), RykDN-GFP (B), RykICD-GFP (C). The first row used antibody to stain against the streptactin domain on the Tandem Affinity Purity (TAP) tag to show that Pcdh19 was expressed in the appropriate cultures. The second row used antibody to stain against the GFP tag on Ryk, and RykDN, and RykICD to show an interaction or lack there of between Pcdh19 and the Ryk construct. (B) Exposure was blown out to better show the interaction band.

that Pcdh19 does interact with Ryk through its extracellular domain (Fig 2). Because Ryk was previously shown to bind to many proteins, extremely bright bands due to lots of binding were not expected (Fig 2). Many attempts were done to improve the cleanliness and definition of the co-immunoprecipitations, but to no avail. Failure to optimize the co-immunoprecipitations prevented proceeding cell fractionation experiments to further understand Pcdh19's influence on Ryk-mediated signaling.

Immunostaining was performed to understand how Pcdh19 affects Ryk-mediated Wnt signaling. HEK cells were transfected with the Ryk constructs alone, or in conjunction with Pcdh19, Wnt3, a ligand involved in neuronal differentiation, or all three. Nuclei were illuminated

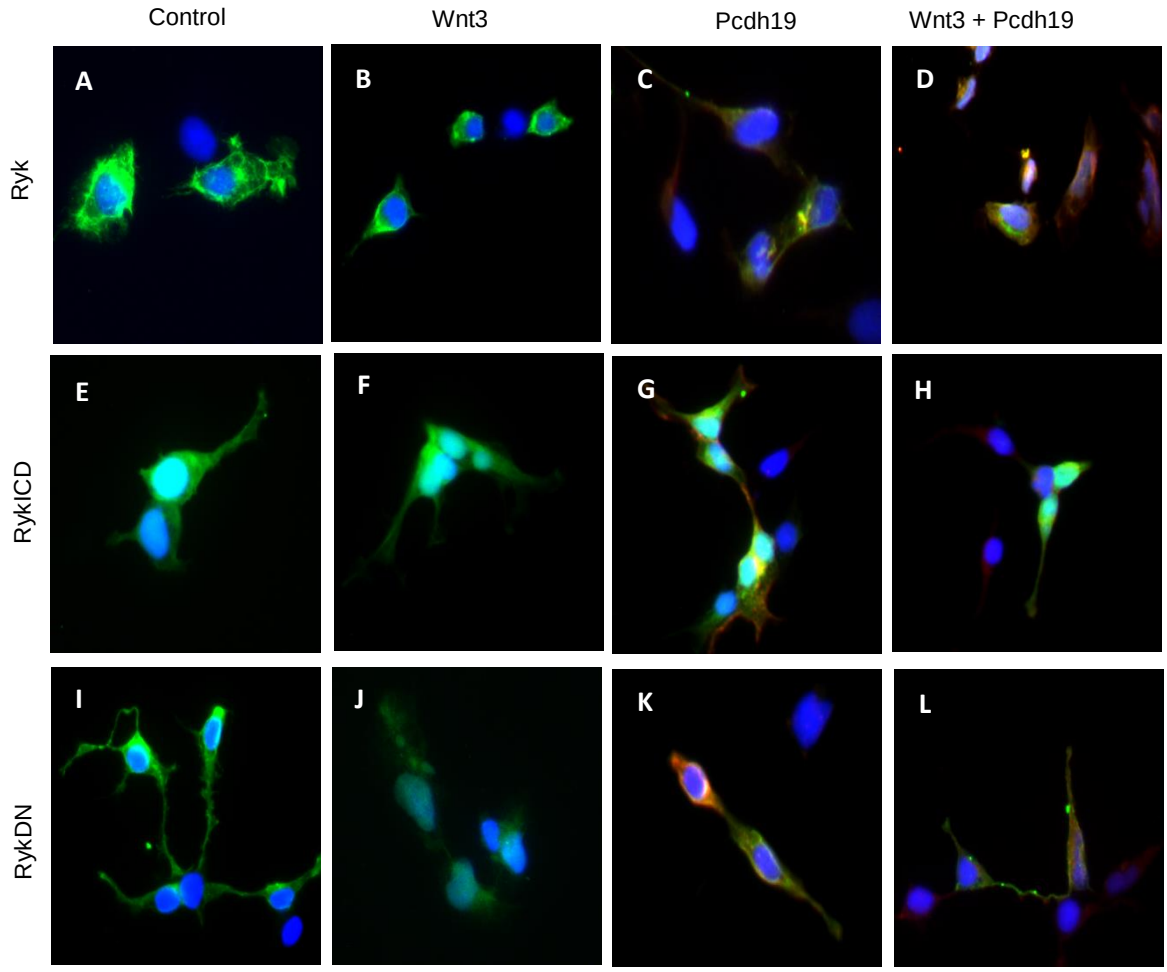


**Fig. 3 Immunohistochemistry to elicit and confirm Ryk mechanism.** HEK293 cells transfected with the indicated genes. GFP tags on Ryk constructs and myc tags on Pchd19. Anti-rabbit antibody conjugated with Dylight 488 interacted with anti-GFP to illuminate the Ryk constructs as green. Anti-mouse antibody conjugated with Dylight 550 interacted with anti-myc to illuminate the Pcdh19 as red. DAPI stain (blue) shows the nuclei.

using a DAPI stain, and α-GFP and α-myc antibodies were used against the Ryk constructs and Pcdh19 respectively. Secondary antibodies were used to illuminate Ryk constructs in green and Pcdh19 in red. Ryk constructs expressed individually displayed protein as expected. RykDN was localized to the cellular membrane (Fig 2I), and RykICD was localized to the cytoplasm and nucleus. Ryk was predominantly found on the plasma membrane (Fig2E). However, there was minor green expression in the cytoplasm and nucleus, likely resulting from endogenous γ-secretase activity. Because the GFP tag was attached to the C-terminus, fluorescence followed the position of the intracellular domain (Fig 2A). Additionally, Ryk and Pcdh19 cotransfections

indicate that they do interact, and that Pcdh19 inhibits nuclear translocation of the RykICD as evidenced by less green fluorescence in the nucleus between Fig 2A and 2C.

In vitro results were intriguing, but in vivo experiments are needed to confirm mechanisms elucidated in vitro and their place in the grand scheme of neurodevelopment. Oligos from which CRISPR RNAs were created and injected along with Cas9 mRNAs into single-celled embryos. The goal was to create a Ryk KO and cross them with Pcdh19:Gal4;5xUAS:Lifeact wildtype and Pcdh19 KO zebrafish to study the effects of neuronal proliferation, differentiation, and columnar organization. F0s were screened using PCR of the CRISPR site, then a test digest with XmaI which had a cut site within the CRISPR site. The PCR band would be protected if the CRISPR worked, or the band would be cut in half if unsuccessful. This method demonstrated that CRISPR worked to some extent. At least, some fish appeared to be mosaic Ryk KOs. If the mutation occurred in the germline, then it could be passed to the following generation. High resolution melt analysis (HRMA) also seemed to indicate that it worked. However, genotyping in the F1 generation was not promising. Both the digests and HRMA seemed inconclusive at best. Multiple efforts resulted in the decision that the CRISPR was unsuccessful. A Ryk BAC was being developed, but this endeavor was dropped when it was known that a Ryk KO zebrafish couldn't be achieved in a timely manner.

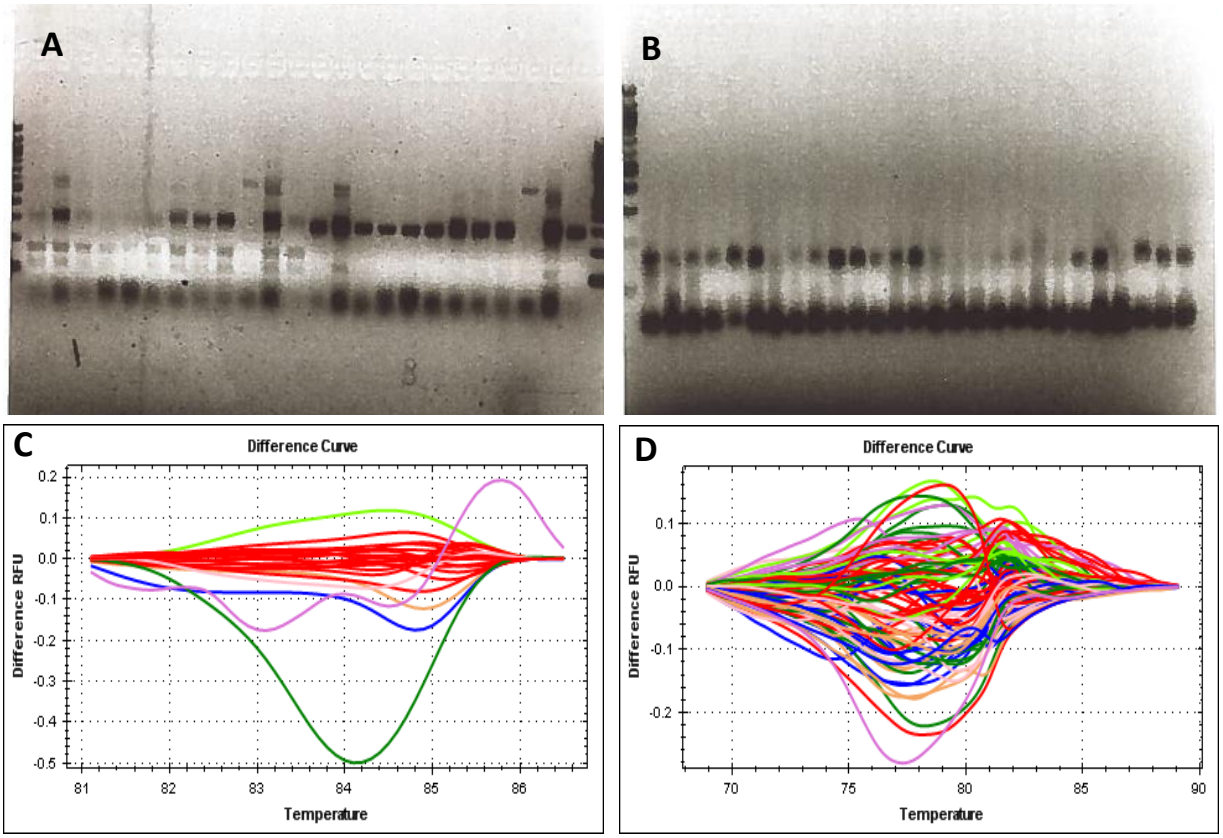


**Fig. 4 Using CRISPR against Ryk to make a knockout line with which to do in vivo studies. (A,B)** Test digests of PCR amplicons containing the Ryk CRISPR site for F0 and F1, respectively. F0 appeared to work, but F1 did not appear so. In A, the first half of wells represent test digests, and the rest are the PCRs before the digests. **(C)** HRMA of zebrafish injected with a CRISPR against Ryk. The red lines indicate wild types and injected fish that didn't not receive a genetic lesion. The deflections indicate that the CRISPR worked in some fish. **(D)** HRMA of F1 generation of fish that have Ryk CRISPR'ed. The results are messy and confirm that the CRISPR didn't work.

Because protocadherins could act similarly and be responsible for a combinatorial code that assemble stereotyped connections and other protocadherins have been shown to interact with Ryk, we performed phospho-histone H3 staining to compare proliferation between protocadherin 18b (Pcdh18b) knockouts and wildtypes. Wildtype and mutant progeny were stained at 24 hpf. There was a statistical difference between the two categories where Pcdh18b mutants displayed slightly more mitotic events indicative of proliferation, although these results are not noticeable by mere looking at the images (wild type: 129.75±7.96, n=12; mutant: 143.56±12.02, n=9; P=0.045, student's t-test).

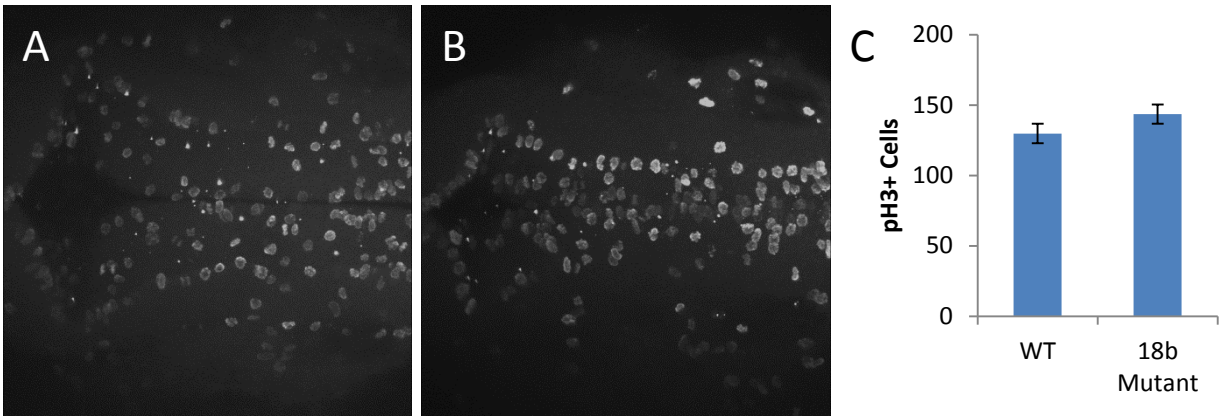


**Fig 5:** Phospho-histone H3 staining. Staining was performed to compare proliferation in WT (A) and Pcdh18b mutants (B). The number of cells in a stack of images were counted as shown by the graph (C). The data indicated that Pcdh18b had statistically more proliferating cells than WT (wild type: 129.75±7.96, n=12; mutant: 143.56±12.02, n=9; P=0.045, student's t-test).

## DISCUSSION AND FUTURE DIRECTIONS

Preliminary experiments showed that Ryk and Pcdh19 do interact. The extracellular domain of Ryk is required for the interaction. Pcdh19 likely binds at Ryk's extracellular domain to inhibit the binding of Wnts, thereby inhibiting Wnt signaling. Immunostaining seemed to support this hypothesis. However, more attempts are required to take more pictures and quantify signals to determine overlap in the membrane or nucleus. Stronger and consistent bands between Pcdh19 and Ryk or RykDN would be desirable. Using confocal imaging during immunocytochemistry would yield clearer results with regards to nuclear translocation. Also, it proved difficult to keep the HEK cells on the coverslip, which led to very few samples to image. Despite numerous efforts and troubleshooting, basic immunostaining and Co-IPs proved difficult. There were many setbacks that prevented the initiation of other experiments, such as cellular fractionation for western blots. Although, lab personnel managed to obtain a clean Co-IP between Ryk and Pcdh18b, adding further weight to the idea that there is a molecular relationship between protocadherins and Ryk (unpublished and not pictured).

Phospho-histone H3 staining of wildtype and Pcdh18b mutant zebrafish showed modest but statistically significant increases in the number of proliferating cells. As stated earlier, it

appears that other protocadherins regulate proliferation. Similar experiments in zebrafish of various protocadherin knockouts would support or reject this hypothesis. Given the overlapping spread in data between the wildtypes and Pcdh18b mutants, it would probably be worthwhile to repeat the experiment to more clearly show a difference in proliferation, despite already establishing statistical significance.

After further investigation, it was determined that the first CRISPR failed because it was designed improperly. The PAM site was mistakenly included in the gRNA. The PAM site was to remain just downstream of the CRISPR site and not part of the site itself. A new CRISPR site has been chosen and development of the Ryk knockout is underway.

Although many of the experiments didn't work, the hypothesis that Pcdh19 interacts with Ryk to regulate neuronal proliferation should be pursued further. Because Ryk has been shown to interact with other protocadherins, this mechanism could be a significant factor in total proliferation in the central nervous system, especially in complex, layered brain regions (Berndt, et al., 2011). To save funds and resources, in vitro experiments should be paused. Efforts should be directed at pursuing a Ryk knockout zebrafish line. Ryk knockout fish and be crossed with transgenic lines of different protocadherin knockouts, including a Pcdh19 knockout. If the mutant phenotype is rescued in the progeny, then it confirms that the Pcdh19-Ryk interaction is crucial for neurogenesis and columnar organization.

In the meantime, it would be worthwhile to revisit the EFMR disease mechanism from another perspective. Previously, the lab described a cis complex between Pcdh19 and N-cadherin (Ncad) that significantly enhance the homophilic adhesive capabilities of Pcdh19 (Emond, et al., 2011). The Pcdh19-Ncad complex only binds to other Pcdh19-Ncad complexes via Pcdh19

homophilic binding. The complex does not bind to Ncad itself, nor does the Pcdh19-Ncad complex bind to complexes of N-cadherin and other protocadherins.

Previously, this complex was studied to determine a mechanism for neurulation defects (Biswas, et al., 2010). Research had shown that mutant Pcdh19 or mutant Ncad led to similar malfunctions in neurulation and brain morphogenesis. Emond, et al. (2011) acknowledged the lack of a Pcdh19-Ncad complex using Pcdh19 mutants seen in EFMR patients as a functional defect of EFMR. However, there have been no other results or findings in the literature that elicit the molecular mechanism for EFMR, other than the aforementioned benefits of corticosteroids. Numerous papers hypothesize that cellular interference as a result of X-inactivation leads to a scrambled signal that manifests itself as epilepsy (Redies, et al. 2012; Harssel, et al., 2013; Dibbens, et al. 2008; Leonardi, et al., 2014). Again, pathways responsible are unknown.

It is possible that the mechanism for cellular interference due to X-inactivation is actually known. Imagine a field of cells, like the one below (Fig 6). In females with two normal copies or males with one normal copy, cells would likely adhere via a Pcdh19-Ncad complex (Fig 6A). In females with two mutant copies or males with one mutant copy, the cell may adhere via Ncad-Ncad interactions or other adhesive proteins (Fig 6B). In the case of EFMR patients in which a female has one normal copy and one mutant copy, some cells would have Pcdh19-Ncad cis complexes, and others would not (Fig 6C). If cellular adhesion behaves similarly to *in vitro* experiments, some cells would adhere together via the Pcdh19-Ncad complex, but other cells could not adhere because Ncad or Pcdh19 alone does not exhibit strong adhesion with the cis complex. Theoretically, half the developing neurons would be unable to bind to each other or demonstrate only weak adhesion. Cellular migration likely requires strong adhesion amongst cells. The lack of strong connections due to random cells expressing mutant Pcdh19 could

disrupt migration seen during neurulation or when neurons migrate along radial glia to mature positions (Biswas, et al. 2010; Cooper, et al; 2015).

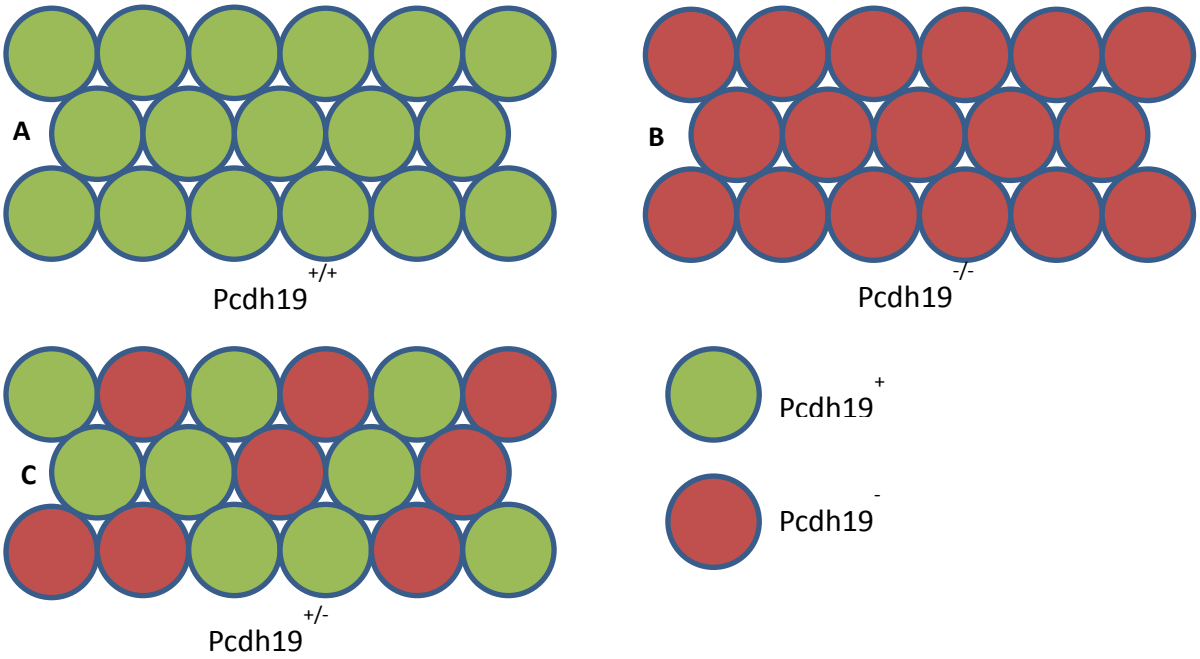


**Fig. 6: A schematic of X-linked inactivation as it pertains to expression patterns. (A, B)** Normal or mutant Pcdh19 will be expressed ubiquitously in homozygotes, even with X-linked inactivation. **(C)** Heterozygotes for Pcdh19 will result in individual cells stochastically expressing either form due to X-linked inactivation, leading to mosaicism in neuronal tissue.

The next step is to determine the downstream effects of failed adhesion due to the decrease in Pcdh19-Ncad complexes and how they relate to EFMR. The trick to relating findings to EFMR is that results must be unique to zebrafish that are heterozygotes for mutant Pcdh19 or mosaically express Pcdh19. Since increased neuronal proliferation was discovered in Pcdh19 heterozygotes and knockouts, proliferation is likely not the only mechanism that leads to EFMR symptoms. It may play a part and still demonstrates Pcdh19's role in neurogenesis, but there are likely other pathways that contribute to the manifestation of the disease. Previously, morpholinos were used against Pcdh19 to study neurulation (Biswas, et al., 2010). Since morpholinos can have a varied effect, the published results were possibly due to mosaic expression. Similar experiments should be conducted in Pcdh19 knockouts to see if there are similar defects in neurulation. If not, then faulty neurulation during development may lead to EFMR.

The proliferation phenotype is also very significant and should be investigated in its relation to the Pcdh19-Ncad complex. The literature shows that cadherin-mediated adhesion is critical for regulating neurogenesis through the Notch-Delta pathway (Hatakeyama, et al. 2014). Using a DN-cadherin to disrupt adherins junctions, they showed an increase in the number of neurons, similar to the results shown by Cooper et al. (2015). Mosaicism of Pcdh19 mutants may lead to faulty adhesion at adherins junctions. Without proper contact between cells, progenitors and neurons would be unable to maintain cell-to-cell signaling through Notch, which would lead to increased neurogenesis due to the lack of inhibitory signal.

It's also worth noting that N-cadherin binds to β-catenin which complexes with α-catenin which binds to the cytoskeleton to support N-cadherin mediated adhesion (Aberle, et al., 1996; Gooding, et al., 2004; Huang, et al., 2014; Miyamoto, et al., 2015). β-catenin is also a well-characterized component of the Wnt/β-catenin signaling pathway which regulates proliferation (Angers and Moon; 2009; Kikuchi, 1999; MacDonald, et al., 2009). Before conducting a literature review, the general hypothesis was that lack of N-cadherin mediated adhesion, such as would be seen in a cell expressing only N-cadherin tried to connect with a cell expressing a Pcdh19-Ncad complex, would lead to increased neuronal differentiation. Although there was promise to this idea, it became apparent that this multistep pathway was rather complicated.

Initially, it appeared that adhesion through N-cadherin led maintenance of a progenitor phenotype (Miyamoto, et al., 2015. One mechanism was through the activation of AKT which phosphorylated β-catenin at the Ser-522 residue and phosphorylated GSK3β (Zhang, et al., 2013). This species of phosphorylated β-catenin went to the nucleus to mediate signaling, and the phosphorylated GSK3β becomes inactivated which allows the accumulation of β-catenin in the cytosol and eventually the nucleus. β-catenin signaling was associated with the maintenance of a

self-renewal phenotype (Zhang, et al., 2010; Noles and Chenn, 2007; Wrobel et al., 2007; Sinevas and Pospelov, 2014). In relation to the canonical Wnt/ β-catenin pathway, there was the possibility that adhesion might lead to cleavage of the intracellular domain that releases β-catenin into the cytosol where it accumulates until it transduces the signal. N-cadherin actually forms a complex with presenilin 1 (PS1) in addition to β-catenin (Georgakopoulos, et al., 1999; Uemura, et al., 2007). Presenilin 1 is the catalytic component of γ-secretase which also cleaves Notch and Ryk (Hatakeyama, et al., 2014; Lyu, et al. 2008). Researchers studied PS1 cleavage of Ryk and if that influences neuronal differentiation. In fact, lack of cleavage led to less β-catenin signaling, and there was an increase in neuronal differentiation and migration (Jang, et al., 2011). It appears that adhesion leads to β-catenin signaling and maintenance of a self-renewal phenotype. Adhesion could also lead to cleavage by PS1 to release free β-catenin into the cytosol to further induce signaling. After cleavage of N-cadherin, PS1 may be free to cleave Notch to add redundancy to the signal for a self-renewing precursor phenotype (Georgakopoulos, et al., 1999). If adhesion by N-cadherin induced cleavage by PS1, it would be reasonable to hypothesize that Pcdh19 affects neuronal differentiation and migration through its complex with Ncad. When some cells don't have Pcdh19 to form the Pcdh19-Ncad complex, then cells would lack adhesive contacts which would reduce Notch and β-catenin signaling, leading to differentiation and migration.

However, there are more pieces to the puzzle. Before PS1 can cleave the intracellular the domain, ADAM10, a metalloprotease, must cleave and shed the extracellular domain (Saftig and Lichtenthaler, 2015; Reiss, et al., 2005; Malinverno, et al., 2010; Uemura, et al., 2006). When this occurs, cells demonstrate less adhesion (Reiss, et al., 2005). Eliminating cleavage activity leads to stronger adhesion and more β-catenin localized at the cell membrane. These findings

conflict with what was already discussed (Zhang, et al., 2010; Noles and Chenn, 2007; Wrobel et al., 2007; Sinevas and Pospelov, 2014). Ideally, adhesion would lead to more intracellular cleavage and less localization of β-catenin at the plasma membrane to maintain a self-renewal phenotype. If the conflicting results are correct, then increased adhesion would lead to less β-catenin signaling and more differentiation. This process is contrary to what was previously outlined. The cause for ADAM10 cleavage of Ncad during development wasn't found in the literature, but it was mentioned that NMDA receptor stimulation would induce Ncad cleavage by ADAM10 (Maliverno, et al., 2010; Marambaud, et al., 2003). It was proposed that this might somehow be involved in learning, although it is unclear how the elimination of contact following a signal would improve learning. It should also be noted that in more mature neurons, Ncad is found at synapses (Basu, et al., 2015; Jontes, et al., 2004; Maliverno, et al., 2010; Marambaud, et al., 2003, Georgakopoulos, et al. 1999). The inability of synapses to form between cells with Pcdh19-Ncad complexes and cells with only functioning Ncad may somehow be responsible for the epilepsy in EFMR patients, especially if additional functions are linked to NMDA receptors. However, this possibility wasn't really explored while reading the literature because it likely does not regulate neurogenesis.

Another paper had some strange findings. Overexpressing full-length or a dominant-negative N-cadherin without the extracellular domain led to more differentiation and less precursors, which was shown to be the result of less β-catenin signaling (Noles and Chenn, 2007). This indicates that β-catenin signaling is independent of adhesion but related to the amount of Ncad. Interestingly, these researchers distinguished that the decrease in β-catenin signaling resulted in an increase in neuron-neuron daughter cells, as opposed to precursor-

precursor or precursor-neuron proliferation. More specifically, there was an increase in the number of daughter intermediate precursors and a decrease in the number of daughter radial glia.

Trying to uncover Ncad's role in proliferation is not very clear and is even contradictory at times. However, adhesion or the lack of adhesion by Ncad due to stochastic expression of normal or mutant Pcdh19 could affect signaling pathways that regulate proliferation and differentiation and explain the lab's prior results. However, the literature review elucidated the need for future research to be very thorough and consistent with definitions and experimental parameters. For example, an observed increase in proliferation or neurogenesis could be the result of increasing the number of precursors, or it could be due to early cell cycle exit into a mature neuronal phenotype. These two possibilities are the result of different pathways or opposite results of the same pathway. Lack of stringency with terms can lead to confusion due to apparent conflict among the findings in various labs. Also, cell types need to be more accurately defined. As a progenitor or neuron progresses, minor nuances in its phenotype can change so that a single mechanism can have varying results at different time points (Gotz and Huttner, 2005; Miyamoto, et al., 2015; Noles and Chenn, 2007; Huang et al., 2015). Studies of the evolution of mechanisms over time, especially during development, would likely be useful. One review article admitted that β-catenin could have opposite effects on development depending on the stage of development in which it's studied (Sineva and Pospelov, 2014).

Another area of potential interest is how cell polarity can regulate polarity. Cell-fate as a result of symmetric and asymmetric divisions are based in the concept of cell-polarity. Some research suggests the importance of cell-polarity on how adherens junctions regulate proliferation or differentiation (Gotz and Huttner, 2005; Miyamoto, et al., 2015). Perhaps, Pcdh19 mutants could disrupt these mechanisms (Compagnucci, et al., 2015). However, there is

the possibility that the proliferative phenotype in PCdh19 KOs is totally independent of a contact-dependent mechanism. If there is no functioning PCDH19, then adhesion among all cells would presumably act through N-cadherin, which is potentially why there are no symptoms in Pcdh19 negative males and females. However, this assumption has been untested and should be answered before investigating the mechanisms above. It's possible that mutant Pcdh19 can form cis complexes with N-cad but can't adhere to other complexes which could lead to a proliferative phenotype. Alternatively, Pcdh19-regulated neurogenesis could be due to soluble factors that initiate signals, such as Ryk-mediated Wnt-signaling. The intracellular domain of Ryk was shown to bind to β-catenin, which could explain Ryk's role in Wnt signaling (Berndt, et al., 2011; Tourette, et al., 2014)

Another reason to revisit the Pcdh19-Ncad complex is that it may serve as a model for the manner in which protocadherins may serve as a combinatorial code (Krishna, et al., 2011). Ncad would act as a co-adhesive protein through which signals related to neurogenesis or synaptogenesis could be propagated. The protocadherins act as discriminating adhesive protein that will only bind homophilically and guides neurons to connect only with the appropriate partners. There are so many potential questions to be answered to better understand neurogenesis through the lens of protocadherins and cadherins and to decipher the molecular mechanism behind EFMR. This research has been incredibly interesting due to its novelty and huge implications on major neurodevelopmental processes of which current knowledge only has a fractured understanding.

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